Supporting Information for

“Insight into the Structure and the Mechanism of the Slow Proton Transfer in the GFP Double Mutant T203V/S205A”

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Figure S1: RMSD of T203V/S205A and T203V/S205V.
Figure S2: RMSF of T203V/S205A and T203V/S205V.
**Figure S3:** The steady-state emission of wt-GFP in H₂O and D₂O. This figure shows that the KIE of the wt-GFP is ≈5.
Figure S4: The average number of water molecules around each side chain Cβ carbon (within 4 Å) of Glu222 for the GFP double mutant T203V-S205A along the MD simulations.
Figure S5: The average number of water molecules around each side chain Cβ carbon (within 4 Å) of residues along the two β-strands in the “hole” domain for the GFP double mutant T203V-S205A along the MD simulations.
Figure S6: The Cα backbone-backbone distances between two opposite residues in the two β-strands for the GFP double mutant T203V-S205A during the MD simulations.
Figure S7: RMSD of the fragments of the two β-strands in the “hole” domain of the GFP double mutant T203V-S205A along the MD simulations.
Figure S8: The distance between the O atom of the hydroxyl phenol group of the chromophore and the nearby O atom of the water molecule during the MD simulations. Two water molecules that are replaced during the simulations of 60 ns for T203V/S205A.
Figure S9: The average number of water molecules around each side chain Cβ carbon (within 4 Å) of residues along the two β-strands in the “hole” domain for the wt-GFP.
Figure S10: The average number of water molecules around each side chain Cβ carbon (within 4 Å) of residues along the two β-strands in the “hole” domain for the GFP double mutant T203V/S205V.
**Estimation of pKₐ of Asn146**

It is extremely difficult to estimate the "apparent pKₐ" of a given amino acid in a protein, because in addition to factoring in changes due to the local side-chain environment, one has to average over all the possible charge states of all interacting titratable groups, as a function of pH, in order to determine long-range effects.

However, it is possible to estimate the shift in "intrinsic pKₐ" for Asn146, due to its immediate environment. We used the solvation/desolvation thermodynamic cycle procedure and the APBS package¹⁻³ to estimate the change in Asn146 intrinsic pKₐ. The shift values that were obtained are positive and between 0.2 and 1.0, depending on the particular force field used to assign partial atomic charges (CHARMM, AMBER and PARSE). All other parameters were left at the recommended values¹⁻³. Therefore, one can add to the textbook value for the Asn side chain in solution (16-17) this small value to roughly estimate the Asn146 pKₐ to be greater than 16.

As an independent check of this result, we further estimated the side chain pKₐ of a hypothetical mutation, Asn146 to Asp146 *in silico*, using the programs PROPKA⁴⁻⁷ and PDB2PQR⁸⁻⁹ (which use completely different procedures to estimate pKₐ values) and the result agrees with that obtained from APBS for Asn146. The pKₐ of the hypothetical Asp146 is estimated by PROPKA to be 4.3, and is thus elevated by less than one unit compared to the model amino acid side chain, assumed by PROPKA to be 3.8.

In summary, as expected because of the overall large negative charge on GFP (about -7 at pH 7.0) and the immediate environment of Asn146, there is no possibility that Asn146 is charged under ordinary circumstances.

**References**


